Conjugated Compounds in Cow's Milk

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Fourteen compounds existing in cow's milk as conjugates (*e.g.*, glucuronides and sulfates) were identified. The compounds found were C_8, C_{10}, C_{12}, and C_{16} normal aliphatic acids, C_{12} γ-lactone, C_{10} and C_{12} δ-lactone, *p*-cresol, 4-ethylphenol, indole, vanillin, ethyl and methyl vanillate, and *p*-hydroxyacetophenone. The conjugates were isolated by adsorption on a neutral resin and then hydrolyzed enzymatically. Gas–liquid chromatography and mass spectrometry were then used to identify the compounds. It is speculated that the origin of some of the free flavor compounds arises from the action of enzymes normally present in milk on the conjugates.

In another work (Brewington *et al.*, 1972), in which the absence of conjugated sex hormones in cow's milk was demonstrated, it was observed that many compounds apparently do exist in milk as conjugates, presumably detoxification products; *e.g.*, glucuronides and sulfates. Only Spinelli (1946) has found a conjugate in cow's milk, and that was indoxyl sulfate (indican). However, Heyns *et al.* (1956) did discover glucuronic acid other than in its free form in human milk, indicating that glucuronides could occur in milk. The purpose of this investigation was to characterize the structure of the conjugates previously observed by us.

ISOLATION OF CONJUGATES

The procedure for the isolation of the conjugates was, with modification, that described previously (Brewington *et al.*, 1972). Approximately 2250 g of Amberlite XAD-4 (Rohm & Haas Co., Philadelphia, Pa.; trade names are mentioned for identification, implying no endorsement) were slurried in water and poured into a glass column (10 × 108 cm). The column was prepared for use as before. One-hundred liters of raw skim milk (from mixed herd, Agricultural Research Center, Beltsville, Md.) were passed through the column at a flow rate of 200–250 ml/min. After all of the milk had entered the column, the column was washed with redistilled water until the eluate was clear. The material adsorbed was eluted using 10–12 l. of redistilled methanol (Baker Analyzed, Baker Chemical Co., Phillipsburg, N. J.). The methanol solution was evaporated to near dryness in a vacuum flash evaporator and the residue was dissolved in 50 ml of water, which was extracted with 3 × 50 ml portions of redistilled methylene chloride (Baker Analyzed, Baker Chemical Co., Phillipsburg, N. J.). Each 150-ml portion was back extracted each time with 50 ml of water (discarded) and then evaporated to dryness on a steam bath under nitrogen. The residue was dissolved in 100 μl of methylene chloride and 1 μl of this solution was then analyzed by glc. This process was repeated until no material was detected by glc.

ENZYMATIC HYDROLYSIS OF CONJUGATES

The water-soluble material was adjusted to pH 4.5 with an acetate buffer and 2 ml of methylene chloride were added. The mixture was then incubated with shaking at 37° for at least 24 hr with β-glucuronidase (40,000 units) and aryl sulfatase (20,000 units) (Calbiochem, La Jolla, Calif.). After incubation, the free compounds were extracted with 3 × 50 ml of methylene chloride, which were back extracted once with 50 ml of H_2O. The methylene chloride solution was evaporated to dryness under a stream of nitrogen and the residue was redissolved in 100

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Table I. Compounds Identified Existing as Conjugates in Cow's Milk

Compound	Trap no.	Mass spectrum, *m/e* (relative intensity)
Caprylic acid	1	144 (M^+) (1), 129 (1), 115 (7), 101 (15), 87 (10), 85 (17), 73 (68), 69 (11), 60 (100), 55 (31)
p-Cresol	2	108 (M^+) (86), 107 (100), 91 (6), 90 (9), 79 (19), 77 (25), 53 (12), 51 (12)
4-Ethylphenol	3	122 (M^+) (31), 107 (100), 108 (8), 91 (5), 77 (15), 65 (5), 39 (9)
Capric acid	5	172 (M^+) (3), 143 (5), 129 (27), 115 (9), 101 (6), 87 (14), 73 (100), 71 (30), 69 (19), 60 (90), 83 (13), 57 (42), 55 (40)
δ-Decalactone	6	170 (M^+) (not discernible), 152 (3), 114 (10), 100 (8), 99 (100), 71 (45), 70 (32), 55 (36)
Indole	7	117 (M^+) (100), 108 (9), 116 (9), 90 (46), 89 (29), 63 (14), 58.5 (10)
Lauric acid	9	200 (M^+) (6), 171 (5), 157 (6), 143 (5), 129 (24), 115 (13), 101 (11), 97 (10), 87 (15), 83 (16), 73 (100), 71 (26), 69 (25), 60 (91), 57 (48)
γ-Dodecalactone	10	85 (100), 56 (11), (spectrum very weak).
δ-Dodecalactone	11	198 (M^+) (not discernible), 180 (2), 114 (11), 99 (100), 71 (37), 70 (30), 69 (19), 57 (15), 56 (18), 55 (48)
Vanillin	11	152 (M^+) (98), 151 (100), 137 (10), 123 (20), 109 (25), 81 (32)
Methyl vanillate	12	182 (M^+) (51), 151 (100), 123 (20), 111 (9), 108 (8), 99 (7), 79 (7), 77 (7)
Ethyl vanillate	13	196 (M^+) (41), 181 (5), 168 (18), 153 (10), 152 (13), 151 (100), 123 (18), 108 (8)
Palmitic acid	18	256 (M^+) (14), 227 (4), 213 (14), 185 (8), 173 (5), 171 (8), 157 (9), 129 (32), 73 (100), 60 (85)
p-Hydroxyacetophenone	19	136 (M^+) (33), 121 (100), 122 (9), 93 (24), 65 (25)

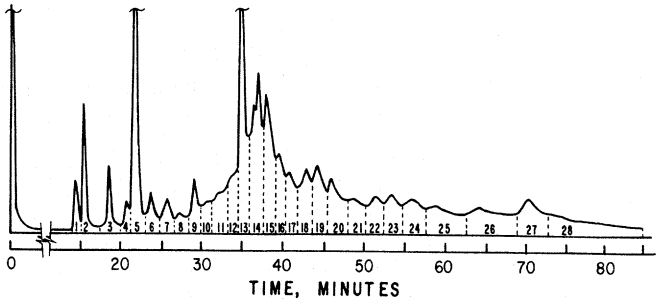

Figure 1. A typical gas–liquid chromatogram of compounds found after hydrolysis of isolated conjugated material. For conditions, see experimental data.

μl of methylene chloride, of which 1 μl was analyzed by glc.

GAS–LIQUID CHROMATOGRAPHY (GLC)

The chromatograph employed was the Perkin-Elmer 900, equipped with a flame ionization detector. The column was a ⅛ in. × 5 ft stainless steel column packed with 7.5% ethylene glycol adipate (EGA) and 2% H_3PO_4 on 90–100 mesh Anakrom ABS. The carrier gas was helium at a flow rate of 30 ml/min. The injection port and detector temperatures were 210°. The column in all cases was programmed between 90 and 190° at 2.5°/min.

Certain areas, as shown in Figure 1, were trapped in glass capillary tubes cooled with Dry Ice by repeated injections of 4–5 μl of the final extract until enough was obtained for analysis by mass spectrometry. The conditions used were the same as described.

GLC–MASS SPECTROMETRY

The mass spectra were obtained using an LKB-9000 spectrometer connected to a gas chromatographic column. A 10-ft EGA–H_3PO_4 column as previously described was used for the analysis. Other operating conditions were: flow, 20 ml/min of helium; separator, 240°; injection port, 240°; and ion source, 290°. The spectra were obtained at a constant accelerating voltage of 3500 V and an electron energy of 70 eV.

RESULTS AND DISCUSSION

A typical chromatogram of the material found after enzymatic hydrolysis is in Figure 1. The pattern obtained for each analysis made was very similar; however, differences in relative peak heights did occur between analyses. The total amount of the free compounds found after hydrolysis was 20 mg (0.2 ppm).

Table I lists the compounds found, the area trapped (trap no.), and the most important mass spectral *(m/e)* peaks. *M/e* values below 50 were not included unless they were of diagnostic value. The compounds were identified by comparison of their retention times and mass spectra with authentic compounds.

All of the compounds identified, except for ethyl and methyl vanillate, *p*-hydroxyacetophenone, and 4-ethylphenol, have been found in milk systems before and contribute to the overall flavor picture. Since there are aryl esterases (Kitchen, 1971) in cow's milk as well as β-glucuronidase activity (Kiermier and Gull, 1966), the possibility of these compounds originating from a conjugated form is not remote. This is important because in many cases the origin of many of the compounds is not known or fully understood. The total amount of released compounds also suggests that conjugates may be a significant source.

The reason why indole is present is not apparent. Though indoxyl sulfate has been reported (Spinelli, 1946) and is the detoxification product of indole, it would not revert to indole upon hydrolysis, but instead would be oxidized to indigo, which would not be detected.

Also of interest is that para substitution prevailed. It is not easy to speculate on the reason, since ortho- and meta-substituted hydroxy compounds are also found conjugated (Williams, 1959).

LITERATURE CITED

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